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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

RECEIVED JAN 28 1974

Date: January 22,1974

1. Principal Investigator (give title and degrees):

George B. Weiss, Ph.D.
Associate Professor of Pharmacology

2. Institution & address:

University of Texas Southwestern Medical School
5323 Harry Hines Boulevard
Dallas, Texas 75235

3. Department(s) where research will be done or collaboration provided:

Department of Pharmacology

4. Short title of study:

Agents which Alter the Transport, Storage and Distribution of Nicotine

5. Proposed starting date: January 1, 1975

6. Estimated time to complete: Three years

7. Brief description of specific research aims:

The specific aims for the initial period of requested support are:

a. To examine, in a comparative and systematic manner, the precise mechanisms by which various pharmacological agents alter the transport, distribution or storage of nicotine. Primary attention will be given to use of agents or procedures which inhibit the transport-mediated uptake of nicotine or the intracellular binding of nicotine.

b. To delineate the sequence of events important for the translocation and/or release of biogenic amines by nicotine. Characterization of relevant parameters will be attempted by use of selected agents which (1) directly antagonize the action of nicotine, (2) alter ionic concentrations important for coupling the action of nicotine to that of amine release, or (3) specifically affect the resultant movement of biogenic amines.

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8. Brief statement of working hypothesis:

The working hypothesis for this project is that important actions of nicotine in the central nervous system are based upon specific alterations in intracellular storage, binding and movements of biogenic amines. These effects depend at least partly upon the different ways in which nicotine is accumulated and concentrated in brain tissue. More specifically, there appears to be not only a passive distribution of nicotine in accord with cellular pH gradients but also an active energy-dependent uptake and retention of nicotine in various brain areas. The interactions between nicotine accumulated in this manner and endogenous stores of biogenic amines present in some brain areas provide a logical focal point for examination of the mechanisms by which central effects of nicotine are induced. In an analogous manner, characterization of the specificity of transport system responsible for a portion of the nicotine accumulation should provide information about the type or types of agents which either utilize or inhibit this process.

9. Details of experimental design and procedures (append extra pages as necessary)

a. General Procedures

Brain sections are obtained from male Wistar rats weighing 180-250 g. All animals are decapitated while under light ether anesthesia, brains rapidly removed, and brain areas prepared for slicing by dissection on filter paper which has been moistened with bathing solution and placed on ice. The general dissection procedure has been described (1). The specific procedure to be used involves preparation of two slices from each of 3 different brain areas. First, one slice is cut from the lateral surface of the cortex of each hemisphere. Second, the corpus callosum is removed, the two striatum sections dissected out, and each striatal portion is sliced in half with the two halves considered as a single slice. Third, the brain is turned over and the hypothalamus isolated and divided in half at the midline. Each half is sliced in two and the two parts are treated as a slice. Slices (approximately 0.5 mm thick) are prepared with a longitudinal vibrating monofilament nylon thread (2) and incubated at 37°C in a bicarbonate-buffered salt solution at pH 7.3.

In experiments measuring uptake and washout of ^{14}C-labeled compounds, the procedures employed would be similar to those described for ^{14}C-serotonin in smooth muscle (3,4), ^{14}C-nicotine in striated muscle (5-7) and brain area slices (8), and ^{14}C-serotonin in brain area slices (9). For uptake measurements, slices are placed in tubes containing aerating bathing solution plus radioisotope. After the desired incubation interval, slices are removed from the radioactive solution, dipped rapidly into two consecutive tubes containing nonradioactive solutions similar to the incubation solution (to remove radioactive bathing solution adhering to the surface of the slices), and drained by gently rolling on a glass surface until essentially all adherent solution is removed. Then slices are weighed on a Federal-Pacific torsion balance (sens. 0.05 mg) and placed in test tubes; the ^{14}C-labeled compound is removed by dialysis or extraction. Aliquots from the resultant solution are placed into appropriately prepared planchets, dried and counted in a Nuclear-Chicago proportional gas flow counter. Uptake can be expressed as a tissue space (ml/g) or as tissue uptake (micromoles per gram of tissue).

Washout experiments are similar to uptake experiments in most details. A special holder is employed to facilitate serial transfer of freely floating slices (10). After incubation and dipping, the slices are transferred through a series of test tubes, each of which contains a known volume of aerated nonradioactive medium (11). Upon completion of the washout, each slice is drained by gentle rolling, weighed, and ashed as in the uptake experiments. Curves delineating (a) the decline of tissue radioisotope

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with time, expressing this as a percentage of the initial isotope content (desaturation curve), and (b) the percentage of the remaining isotope washed out of the tissue per minute of each collection interval (rate coefficient curve) were constructed. Both of these plots have been used to describe loss and alterations in loss of radioisotopes (12,13).

Assay procedures employed for quantitative analysis of ^{14}C-serotonin and its metabolites are described in previous studies with isolated smooth muscle (3,4) and brain area slices (9). Quantitative detection of catecholamines (norepinephrine, dopamine, and metabolites) will be accomplished by isotopic (e.g. 14-16) techniques; tissues would be exposed to either ^{14}C-labeled norepinephrine or ^{14}C-labeled dopamine. Separation of catecholamines and their metabolites will be accomplished by a modified method based on that of Neff and Costa (17). Brain area slices would be homogenized in 4 ml of 0.4N perchloric acid and, after centrifugation, an aliquot of the clear supernatant will be taken and total radioactivity determined. To determine the relative proportions of ^{14}C-labeled catecholamines and their metabolites present, the remaining supernatant will be placed in a glass-stoppered centrifuge tube containing prepared alumina (18) and the pH of the mixture will be adjusted to pH 8.2-8.4 with 0.5 M tris buffer. After centrifugation and separation, catechols will be eluted from the alumina with 0.4N acetic acid.

b. Specific Experimental Approaches

An outline of the initial areas of investigation indicated by the specific aims (#7) can be provided. Most of the techniques to be employed have been or are being carried out in this laboratory. Consequently, serious technical difficulties are not anticipated. The initial experiments are designed to reveal the most relevant and accessible relationships in as efficient a manner as possible.

Specific aim "a" experimental approaches:

(1) Characterization of effects of appropriate pharmacological agents on transport, distribution and storage of nicotine in slices from different rat brain areas. Initially, effects of various agents on ^{14}C-nicotine efflux and uptake will be ascertained. Attention will be focused on the mechanisms of action of agents known to inhibit uptake of ^{14}C-nicotine. Preliminary experiments show that ^{14}C-nicotine uptake was markedly inhibited by procaine, mecamylamine, physostigmine (but not acetylcholine) and lobeline, and increased by high (depolarizing) potassium ion concentrations. Further experiments with these agents are planned to determine whether some (physostigmine?, lobeline?) may specifically compete with nicotine for intracellular binding sites whereas others (procaine?, high potassium?) may act by altering cell polarization and permeability.

(2) Examination of effects of nicotine analogues and antagonists on ^{14}C-nicotine distribution and accumulation. Following delineation of sites at which ^{14}C-nicotine is taken up and accumulated, it would be practical to examine the actions of nicotine-related agents in the same manner. This group of agents includes nornicotine (a nicotine precursor), cotinine (a nicotine metabolite), nicotine analogues (e.g. nicotine monomethiodide) and nicotine antagonists (e.g. DMAE). It is anticipated that newer nicotine antagonists will be developed in the near future and will be made available to us. For example, Dr. John Long (Univ. of Iowa) has recently sent

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us a supply of a new derivative of DMAE (TP-DMAE) which has no sympathomimetic activity. It would be of interest to examine the effects of this new junctionally-active agent on nicotine-induced effects and distribution in brain area slices.

(3) Development of isolated central and peripheral preparations for measuring and comparing nicotine antagonist activity. The studies on effects of pharmacological agents and nicotine-related compounds on ^{14}C-nicotine in brain area slices would be compared with relevant analogous interactions in isolated peripheral systems in which junctional or ganglionic actions of nicotine can be readily examined (e.g. frog rectus abdominis muscle, guinea pig ileal preparation). It is necessary to distinguish between effects and movements of nicotine important in both central and peripheral systems and those actions of nicotine which are present only in central nervous system tissue. As an example, do the standard nicotine junctional blocking agents (curare, decamethonium) affect brain area distribution of nicotine? Conversely, is atropine a more effective central antagonist of nicotine? Resolving these interrelationships would be necessary to dissociate antagonist activities at different types of nicotinic sites or stores.

Specific aim "b" experimental approaches:

(1) Interactions between nicotine and biogenic amines. Previous studies on the action of nicotine on ^{14}C-serotonin distribution (9) and related preliminary data with ^{14}C-norepinephrine and ^{14}C-dopamine (19) indicate that nicotine has specific effects on the distribution of these biogenic amines in striatum or hypothalamus slices. The critical question is whether these effects of nicotine represent specific interactions between nicotine and a biogenic amine at intracellular sites or, alternatively, represent a sequential reaction in which one or more intermediate steps serve to couple the specific action of nicotine with a translocation of cellular amine. Two approaches will be used initially to clarify this point. First, we will further examine specific effects of nicotine on the cellular distribution and accumulation of appropriate ^{14}C-labeled amines to ascertain whether the amine stores affected are primarily at binding sites within the cell membrane, within intracellular depots (granules?), or at nonspecific intracellular locations. Secondly, we would examine the effects of biogenic amines upon ^{14}C-nicotine accumulation. Presumably, if nicotine and amines interact at specific cellular binding sites, these amines should have effects on ^{14}C-nicotine similar to those which nicotine has on ^{14}C-labeled amines.

(2) Delineation of mechanisms of nicotine-amine interactions. Subsequent to determination of nicotine-amine interactions, we would examine the influence of a variety of parameters which may affect differing aspects of these relationships. These would include duration of exposure (transient or maintained effect?), effects of temperature and metabolic inhibitors (nonspecific energy-dependence of effects?), and actions of ouabain and of variation of ionic concentrations (specific effects on energy-dependent transport?).

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References

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3. Weiss and Rosecrans, Eur. J. Pharmacol. 13: 197, 1971.
4. Weiss and Rosecrans, Eur. J. Pharmacol. 14: 130, 1971.
5. Weiss, J. Pharmacol. Exp. Ther. 154: 605, 1966.
6. Weiss, J. Pharmacol. Exp. Ther. 160: 135, 1968.
7. Weiss, J. Pharmacol. Exp. Ther. 160: 148, 1968.
8. Weiss and Alderdice, MS in preparation.
9. Goodman and Weiss, Neuropharmacology 12: 955, 1973.
10. Arnfred, Hertz, Lolle and Lund-Andersen, Exp. Brain Res. 11: 373, 1970.
11. Hertz, J. Neurochem. 15: 1, 1968.
12. Bianchi, J. Pharmacol. Exp. Ther. 147: 360, 1965.
13. Weiss, J. Pharmacol. Exp. Ther. 154: 595, 1966.
14. Kopin, Axelrod and Gordon, J. Biol. Chem. 236: 2109, 1961.
15. Iversen, Glowinski and Axelrod, J. Pharmacol. Exp. Ther. 151: 273, 1966.
16. Whitby, Axelrod and Weil-Malherbe, J. Pharmacol. Exp. Ther. 132: 193, 1961.
17. Neff and Costa, Life Sci. 5: 951, 1966.
18. Crout, Standard Methods of Clinical Chemistry, Vol. 3, p.62. Academic Press, New York, 1961.
19. Goodman, unpublished data.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

a. Space available: Fully equipped new laboratory facilities suitable for radioisotope and related work.

b. Facilities available:

(1) Two automated Nuclear-Chicago planchet counting systems (one low background).
(2) An Olivetti Programma 101 and several electronic calculators.
(3) Cahn electrobalance, torsion and analytical balances, pH meters, heaters, muffle furnaces, constant temperature baths, recorders.
(4) Custom designed slicer and related equipment for brain slice preparation.
(5) Grass model 7 polygraph with Model 7P1 Low-Level D.C. Preamplifiers and FT.03 strain gauge transducers.

Also in the department and readily available are:

(6) Beckman Model 979 Atomic Absorption-Spectrophotometer system.
(7) A variety of centrifuges including a Beckman L2-65B Ultracentrifuge.
(8) Analytical equipment and facilities for chemical procedures required for isolation and analysis of drugs and their metabolites.
(9) Equipped small animal room.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

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13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
a. G. B. Weiss, Ph.D.	40	none
b. F. R. Goodman, Ph.D.	35	6125.
Technical		
c. Research Technician II (G. Atkins or to be recruited)	100	8724.
d. Secretary - Linda High	25	1725.
Fringes for b, c and d		1217.
Sub-Total for A		17,791.
B. Consumable supplies (by major categories)		
a. Chemicals		350.
b. Glassware		200.
c. Animals (mainly rats, rabbits)		850.
d. Isotope and related supplies		600.
e. Publications, office supplies and related expenses		700.
Sub-Total for B		2700.
C. Other expenses (itemize)		
a. Maintenance - Nuclear Chicago Counter		600.
b. Travel (FASEB and/or ASPET meetings)		550.
Sub-Total for C		1150.
Running Total of A + B + C		21,641.
D. Permanent equipment (itemize)		
a. Incubation baths, heaters, tissue holders and related minor equipment		400.
Sub-Total for D		400.
E. Indirect costs (15% of A+B+C)		3246.
Total request		25,287.

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	18,858	2700	1150	400	3406	26,514
Year 3	19,989	2700	1150	400	3576	27,815

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
a. Interactions of Nicotine with Cellular Transport Systems on Muscle and Nerve	AMA-ERF	$52,358 direct cost	2/1/73-1/31/75
b. Mechanisms of Drug Action in Vascular Smooth Muscle	NIH HL-14775	$65,835 direct cost	9/1/71-8/31/74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
c. Mechanisms of Drug Action in Vascular Smooth Muscle	NIH HL-14775	$166,275 direct cost	9/1/74-8/31/79

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name George B. Weiss

Signature George B. Weiss Date 1-22-74

Telephone 214 (Area Code) 631-3220 (Number) 2268 (Extension)

Checks payable to

Univ. of Texas Southwestern Medical School

Mailing address for checks

Mr. Julius E. Weeks
Univ. of Texas Southwestern Medical School
5323 Harry Hines Boulevard
Dallas, Texas 75235

Responsible officer of institution

Typed Name F. J. Bonte, M.D.

Title Dean, Univ. of Texas Southwestern Medical School

Signature F. J. Bonte Date 1-22-74

Telephone 214 (Area Code) 631-3220 (Number) 601 (Extension)

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